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Systemic cytokine response following exercise-induced muscle damage in humans

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Abstract

Background: Muscle adaptation which occurs following eccentric exercise-induced muscle damage has been associated with an acute inflammatory response. The purpose of this study was to investigate serum interleukin-6 (IL-6), osteoprotegerin and receptor activator of nuclear factor kB ligand (OPG/RANKL) concentrations following muscle damage. We measured changes for several days following muscle damage.
Methods: Ten healthy young males performed an eccentric exercise protocol using their quadriceps. Blood samples were withdrawn before and at 6 h, 2 days, 5 days and 16 days post-exercise. Functional and clinical measurements were performed before, and on days 1, 2, 5, 8, 12 and 16 post-exercise.
Results: The exercise protocol resulted in muscle damage, indicated by changes in biochemical markers. An increase in IL-6 and OPG, and a decrease in RANKL concentrations were seen at 6 h and on day 2 post-exercise; the OPG:RANKL ratio was increased at 6 h post-exercise ($p < 0.05$).
Conclusions: Changes in IL-6 and OPG/RANKL system may represent systemic responses in muscle inflammation and repair processes. However, further studies are needed to elucidate a potential systemic and/or local role of the OPG/RANKL system in skeletal muscle repair.
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Keywords: cytokines; interleukin-6 (IL-6); muscle damage; osteoprotegerin (OPG); receptor activator of nuclear factor kB ligand (RANKL).

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Introduction

Activity such as muscle overload and muscle stretch, or combination of both that occurs in eccentric exercise has been shown to result in muscle damage (1–5). Exercise-induced muscle damage has been associated with disruption of the normal myofilament structures in sarcomeres (6), damage to sarcolemma, loss of fiber integrity and leakage of muscle-specific enzymes and proteins into the blood (7), acute inflammatory response (8–11), delayed-onset muscle soreness and loss of muscle contraction force (12).

Muscle adaptation that occurs following damage has been explained by the classic damage-inflammation-regeneration process (8, 13, 14). It is thought that exercise-induced muscle damage is associated with aseptic inflammation, and the primary purpose of this inflammation is the repair of damaged tissue (8, 11). Coordination between inflammation and regeneration is crucial for successful recovery of damaged muscle (13). Also, much of the acute inflammatory response induced by muscle damage is coordinated by the de novo synthesis of endogenous cytokines that direct inflammatory-related events (15, 16). Cytokines may be produced by a variety of cells including endothelial cells, tissue-resident leukocytes and circulating leukocytes (15, 17). They contribute to specific aspects of acute inflammation and may be characterized as either pro- or anti-inflammatory, based on their predominant action (18).

Osteoprotegerin (OPG) and receptor activator of nuclear factor kB ligand (RANKL) are specific modulators of the immune system (19, 20). However, no previous work has been done to examine the potential role of these endogenous cytokines regarding exercise-induced muscle damage and adaptation. Hence, the aim of our study was to illuminate the changes in interleukin (IL)-6 and the OPG/RANKL system in blood. We measured these changes for several days following eccentric exercise-induced muscle damage in young men.

Materials and methods

Ethical approval

Written informed consent was obtained by all volunteers participating in this study, which has been approved by the Ethics Committee of the National and Kapodistrian University of Athens. All experimental procedures conformed to the Declaration of Helsinki.

Subjects

Ten healthy men (age 25 ± 1.5 years, height 180.3 ± 1.5 cm, body mass 76.8 ± 2.4 kg, body mass index 23.6 ± 0.5 kg/m^2)

participated in the study. The volunteers were physically active, but not accustomed to high-intensity eccentric exercise and had not participated in any type of resistance training or regular exercise regime for at least 6 months before the study. These individuals were free of any musculoskeletal disorders or other lower extremity pathologies. They refrained from taking any medications or nutritional supplements throughout the experimental period. The volunteers were not allowed to perform any vigorous physical activities or quadriceps muscle stretching during the entire experimental period. They were also instructed to maintain their normal diet with no alcohol. On the day prior to, and the day of each blood collection, they were asked to have the same meals.

Experimental design

The volunteers performed a maximal eccentric exercise protocol of the knee extensor muscles with each leg. Post-eccentric exercise, one leg served for the assessment of muscle function (the functional assessment leg, FL). The contralateral leg was used as a control (the control leg, CL) in order to assess any potential training effects of repeated maximal torque testing on the functional measures. The leg used for functional assessment or control was alternated between subjects to account for variability due to dominant and non-dominant legs. At least 10 days prior to start of the study, volunteers were subjected to pre-exercise (PRE) blood collection. Blood samples were then withdrawn from each individual volunteer at 6 h, 2 days, 5 days and 16 days post-exercise. In addition, prior to exercise volunteers completed two familiarization sessions in which they were acquainted with the measurements and the procedures of the testing and the exercise protocols [see details in 'Testing of muscle function' and 'Maximal eccentric exercise protocol' below]. The functional testing protocol consisted of the assessment of maximal voluntary contractile (MVC) isometric torque of the knee extensors, performed before (PRE) and on days 1, 2, 5, 8, 12, and 16 post-eccentric exercise. The time points of blood sampling and functional measurements were chosen to cover a wide period in the regeneration and adaptation processes following muscle damage. In addition, delayed-onset muscle soreness (DOMS) and changes in thigh circumference were also measured. All measures were performed on both legs at each time point of the testing protocol, except for the maximal isometric torque which was performed on the FL and CL before the eccentric exercise and on day 16 post-exercise.

Testing of muscle function

Volunteers were familiarized with the torque testing procedures on an isokinetic dynamometer (Cybex Norm Lumex, Inc., Ronkonkoma, NY, USA) during two visits to the laboratory. During each familiarization session, subjects warmed up for 10 min on a cycle ergometer at 50 W. After this, they performed a number of comfortable submaximal trials of knee extension to become familiar with the procedures of the isometric torque measurements and to learn proper execution of the exercise. During the second familiarization, baseline measurements of the testing protocol (PRE) were performed with the knee extensors of each leg, and baseline measures were reassessed post-exercise as described above. Maximal isometric torque was measured to evaluate muscle function. Subjects were seated upright on the isokinetic dynamometer with the knee joint aligned with the axis of rotation of the dynamometer's lever arm.

Maximal isometric torque

MVC isometric torque of the knee extensors was measured at eight different knee angles, i.e., 15, 30, 45, 60, 75, 90, 105 and 120°, in random order across subjects and across days for each subject. The knee angles used for MVC torque testing were set using the dynamometer's visual display unit after entering a reference knee angle of 0°, which was defined as full knee extension. Each subject performed two maximal voluntary isometric contractions of 3 s duration at each angle. The best trial was used for the MVC isometric torque of the angle.

Peak torque and optimum angle for peak torque generation were assessed using the isometric angle-torque curve. The curve was determined for each subject by fitting a quadratic polynomial curve to the torque vs. knee angle, as described previously (21).

Maximal eccentric exercise protocol

Subjects performed an eccentric exercise routine with the knee extensors of each leg on the isokinetic dynamometer switched to the isokinetic mode. The exercise protocol consisted of two sets of 25 maximal voluntary eccentric (lengthening) muscle actions with a 5-min break between the sets. Subjects were required to maximally resist the forced lengthening of their quadriceps through a range of motion of 130°. This range of motion represented almost full extension (5°) to almost full flexion. Subjects rotated internally the foot to ensure sufficient activation of the vastus lateralis during each muscle action. Each lengthening muscle action was performed at an angular velocity of 30° s^{-1}, lasted for ~4 s and followed by a 15-s rest phase during which the leg was returned passively to the starting position (5°) by the dynamometer motor. The eccentric exercise protocol lasted for a total of ~22 min for each leg. The order of leg being exercised was randomized across subjects. Subjects were instructed to contract as forcefully as possible; strong verbal encouragement was given during all trials.

Muscle torque, power and work during eccentric exercise protocol

To evaluate total mechanical activity of the exercised muscles during the eccentric exercise protocol, peak torque, average power and total work were recorded throughout the exercise routine for each leg.

Thigh circumference assessment

Changes in thigh circumference were used to estimate the amount of intramuscular and subcutaneous swelling post-exercise as an indicator of muscle damage (6, 22). Thigh circumference assessment of each leg was performed at the start of each measurement session and preceded biopsy and/or muscle function measurements. Circumference measurements were taken by the same investigator using a plastic tape measure at the point corresponding to one-third between the spina iliaca anterior superior and the superior pole of the patella. Subjects stood with their thigh muscles relaxed during the measurement period. The accuracy of this measurement is reported to be within 2 mm (22). The points were marked on the subject's skin with semi-permanent ink to ensure consistent placement of the tape measure. Two measurements were made and the average value recorded.

Muscle soreness

DOMS was evaluated for both legs before any contractions were performed in each measurement session. The subjects visually recorded the perceived pain on a visual analogue scale that had a continuous line of 100 mm, with the left end labeled ''no pain'' and the right end labeled ''extremely sore''. Instructions were given to the subjects to rate soreness levels in two ways: (i) during one repetition of flexing and extending the knee joint throughout the entire range of motion and (ii) upon light palpation of the entire knee extensors area (i.e., the muscle belly and distal regions of the quadriceps muscle). Palpation was always performed by the same investigator, with the subjects thigh at rest (22). Subjects marked the scale and the distance from the left end of the scale to the mark was used as the level of soreness. The average of the two values for each subject was used as the criterion score for that day. DOMS was assessed each day of function testing until subjects reported total disappearance of pain.

Blood sampling and serum measurements

Blood samples were collected prior to (PRE) and after eccentric exercise at 6 h, 2 days, 5 days and 16 days. The subjects were at rest for at least 30 min prior to collection of 10 mL of blood obtained from an antecubital vein. Blood samples were allowed to clot at room temperature for 30 min. Serum was collected following centrifugation at 2000 *g* at 4°C for 10 min, stored frozen in 0.5 mL aliquots at –80°C and thawed one time only at the time of analysis. Measurement of serum lactate dehydrogenase (LDH) activity was performed using an automated commercially available kit (Roche Diagnostics, Mannheim, Germany) in a Roche/Hitachi ACN 057 (Roche, Mannheim, Germany) at 37°C. Myoglobin concentrations in serum were determined using an immunoturbidimetric assay (Turbiquant, Dade Behring, Marburg, Germany). Serum LDH activity and myoglobinemia were used as indirect markers of muscle damage (10, 22). IL-6, OPG and RANKL were determined by standard sandwich enzyme-linked immunosorbent assay (ELISA) using commercially available kits (IL-6: Quantikine HS, R&D Systems Inc., Minneapolis, USA; OPG and RANKL: Biomedica, Vienna, Austria) according to the manufacturer's instructions. Color formation was measured with a microplate reader (Versamax, Molecular Devices, Sunnyvale, CA, USA) at 450 nm, and calculations were performed using SoftMax Pro software (Molecular Devices, Sunnyvale, CA, USA). Briefly, 96-well microtiter plates (Costar) were coated with a monoclonal antibody directed against the analyte. Samples and standards were applied and the bound analyte was detected with horseradish peroxidase conjugated to a secondary polyclonal antibody directed against the analyte. Visualization of the presence of the peroxidase label was achieved with a tetramethylbenzidine (TMB) substrate. Color formation was measured with a microplate reader (Versamax, Molecular Devices, Sunnyvale, CA, USA) at 450 nm, and calculations were performed using a SoftMax Pro software (Molecular Devices, Sunnyvale, CA, USA). All samples were analyzed simultaneously, in duplicate, and the results averaged. According to manufacturers, the minimal detection limits of the assays were 0.039 pg mL^{-1}, 0.14 pmol mL^{-1}, 0.08 pmol mL^{-1} for IL-6, OPG and RANKL, respectively. The intra- and inter-assay coefficients of variation (CV) were as follows: 6.9%–7.8% and 6.5%–9.6% for IL-6, 4%–10% and 7%–8% for OPG, and 3%–5% and 6%–9% for RANKL.

Statistical analysis

Changes in measures of function were assessed using a two-way analysis of variance (ANOVA) with repeated measures (SPPS v. 11 statistical package; SPSS Inc., Chicago, IL, USA). A one-way ANOVA with repeated measures over time was employed to evaluate changes in all serum measurements. Significant F ratios for main effects or interaction ($p < 0.05$) were evaluated by comparing the mean values using Tukey's post-hoc tests. Relationships between variables were examined using Pearson's correlation coefficient (r). All data are presented as mean ± standard error of the mean (SEM). Statistically significant changes were considered at $p < 0.05$.

Results

Assessment of eccentric exercise-induced muscle damage

All volunteers reported significant muscle soreness in both legs ($p < 0.01$), which peaked on day 2 post-exercise [42.5 mm (5.1) for the FL and 50 mm (6.7) for the CL]. No significant differences were found between legs ($p > 0.05$).

Thigh circumference was significantly increased post-exercise, with the highest scores occurring on days 2 and 5 post-exercise for both legs. Scores remained elevated up to day 16 on the FL, and gradually decreased from days 8–16 on CL (Figure 1). No significant differences were found in thigh circumference between legs post-exercise ($p > 0.05$).

Classical biochemical markers of muscle damage were elevated post maximal eccentric exercise protocol. LDH activity was increased and remained elevated up to day 5 post-exercise, while myoglobin concentrations were significantly increased at 6 h and on day 2 post-exercise (Figure 2).

Assessment of muscle function following eccentric exercise-induced damage

As expected, a significant ($p < 0.001$) gradual decrease in peak torque [by 37.5% (5.9) for FL and by 33.4%

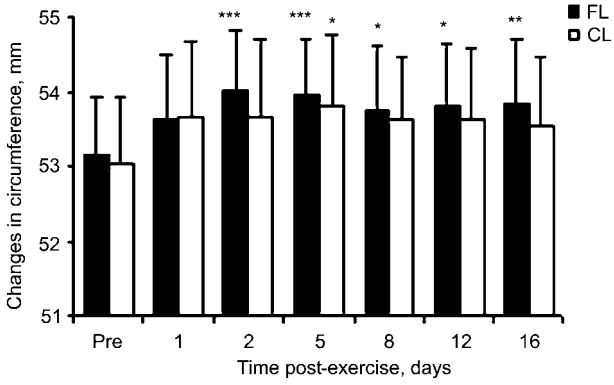

Figure 1 Changes in thigh circumference compared to pre-exercise (PRE) values (mean ± SEM; n = 10).
Thigh circumference was increased post-exercise indicating damage-induced intramuscular and subcutaneous swelling in the thigh. FL, functional leg; CL, control leg. Significantly different from pre-exercise: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

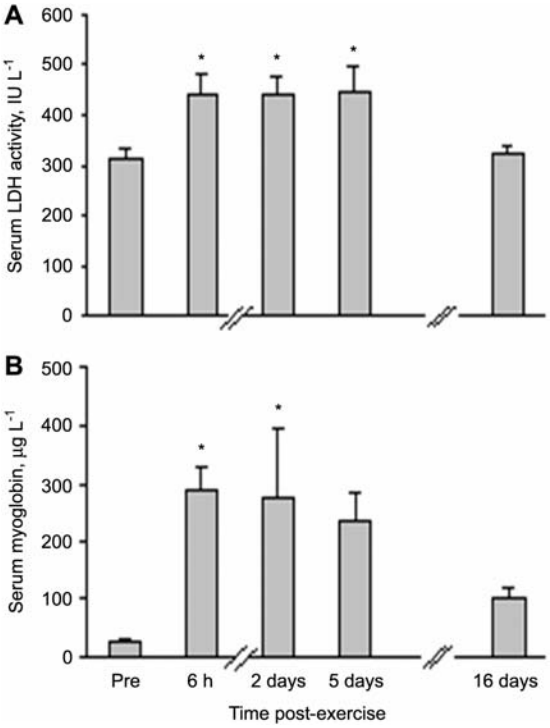

Figure 2 Serum lactate dehydrogenase (LDH) activity (A) and myoglobin concentrations (B) before (PRE) and after maximal eccentric exercise of both legs (mean ± SEM; n = 10).
LDH activity was increased and remained elevated up to day 5 post-exercise. Myoglobin concentrations were increased at 6 h and day 2 post-exercise. *Significantly different from pre-exercise ($p < 0.05$).

(5.7) for CL], average power [by 41.3% (5.0) for FL and by 43.1% (3.0) for CL] and total work [by 42.2% (4.8) for FL and by 41.3% (3.6) for CL] was shown at the end of the course of the eccentric exercise protocol. There were no differences between the two legs in any of the above measures ($p > 0.05$). Similar estimates of the above parameters of muscle function indicate that both the functional assessment (FL) and the control leg (CL) underwent the same effects by the exercise protocol.

Peak isometric torque (Figure 3) was significantly changed over time compared with pre-exercise levels. Further, there was not any training effect of the repeated maximal torque testing on the functional responses of the FL and CL by day 16 post-exercise (i.e., no differences in percent changes of peak torque were found between legs by day 16 post-exercise; Figure 3).

Assessment of cytokine response in blood following eccentric exercise-induced damage

The coefficient r^2 for standard curves of all the ELISA analyses was ≥ 0.98. Analysis of inflammation-related factors in the blood revealed a significant increase in serum IL-6 and OPG, and a decrease in RANKL. In addition, the OPG:RANKL ratio was found to be significantly increased post-exercise (Figure 4). Correlational analyses revealed some noteworthy associations; peak IL-6 was positively related to the peak OPG

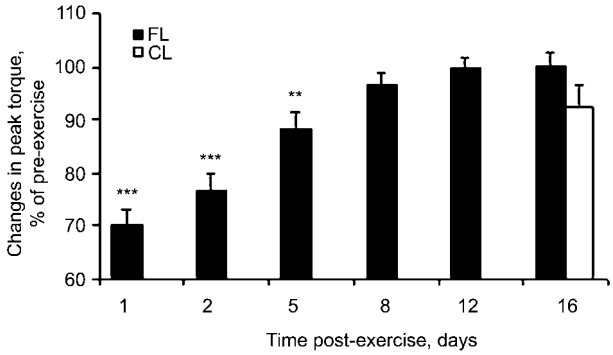

Figure 3 Percent changes in peak isometric torque of knee extensors, calculated using the curve fitting procedure, compared to pre-exercise (mean ± SEM; n = 10).
Peak torque decreased over time compared to pre-exercise levels. FL, functional leg; CL, control leg. Significantly different from pre-exercise: **$p < 0.01$; ***$p < 0.001$.

($r = 0.64$, $p < 0.05$) as well as to the peak OPG:RANKL ratio ($r = 0.90$, $p < 0.05$).

Discussion

The eccentric exercise protocol used in this study resulted in skeletal muscle damage, as indicated by the sustained changes in the clinical markers of damage. Parameters affected included muscle soreness and swelling, a deficit in muscle torque generation and the leakage of muscle proteins into the blood post-exercise.

The molecular mechanisms involved in the muscle repair process following damage include an inflammatory response within the damaged muscle (13, 23). Damage-induced cytokine responses have been demonstrated by increased concentrations of IL-1, IL-6 and IL-10 following high-intensity eccentric exercise (10, 16). Among these, IL-6 is an inflammation-responsive cytokine. Although it has been classified as both a pro- and anti-inflammatory cytokine, the current view is that it acts primarily as an anti-inflammatory factor (18, 24). Our data on inflammation-dependent biomarkers seem to support the concept of a balance between pro-inflammatory and anti-inflammatory factors, which coordinate inflammation and regeneration processes during muscle regeneration (13). An acute inflammatory response appeared to accompany the adaptation process following damage in the present study. This was indicated by the increase in serum IL-6 concentrations within hours post-exercise, corroborating previously published data (10, 16). The early increase in serum IL-6 post eccentric exercise-induced muscle damage has been assumed to originate from inflammatory cells infiltrating damaged skeletal muscle (9, 24). The interactions between muscle and the infiltrating inflammatory cells appear to affect the outcome of the repair process. Cytokines released at the site of damage by activated inflammatory cells and muscle cells act as modulators of the inflammatory process (13). Among those cytokines, it has been suggested that IL-6 controls an excessive inflammatory response following muscle damage, and negatively

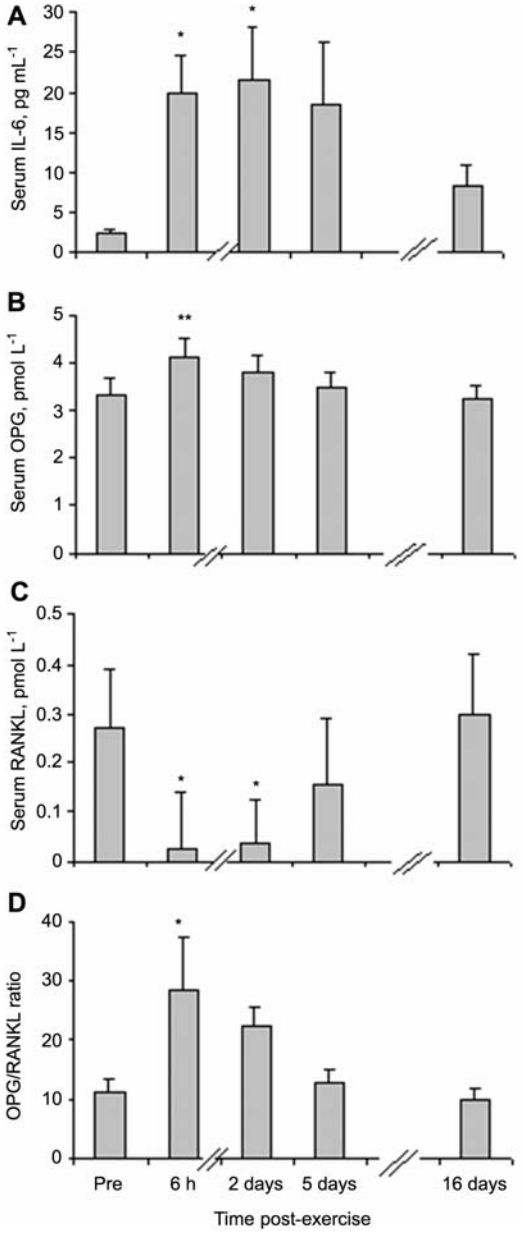

Figure 4 Serum concentrations of (A) IL-6, (B) OPG, and (C) RANKL, and (D) OPG:RANKL ratio before (PRE) and after maximal eccentric exercise of both legs (mean ± SEM; n = 10).
OPG and IL-6 concentrations were increased and RANKL was decreased at 6 h and day 2 post-exercise. The OPG:RANKL ratio was increased at 6 h post-exercise. *Significantly different from pre-exercise ($p < 0.05$).

regulates the acute phase inflammatory response (24). Muscle contractions per se are a stimulus for the production of muscle-derived IL-6 (24–26). However, the kinetics of IL-6 production have shown a progressive increase following muscle-damaging eccentric exercise (10, 16). This is in contrast to the rapid decline of IL-6 concentrations following non-damaging concentric exercise of long duration (24, 26, 27). In agreement with previous findings (16), we found a prolonged increase in IL-6 which could reflect a key role of IL-6 in the repair mechanism after muscle damage (10, 24).

Given that the immune system is modulated by exercise (18), it has been previously proposed that inflammation-related changes following exercise-induced muscle damage could be manifestations of adaptive responses involving immunological events (28). The OPG/RANKL system appears to constitute a complex mediator system involved in the regulation of immune cell function (19). The RANK/RANKL system appears to play a major role in the regulation and function of the immune response (20). Conversely, the RANK/RANKL system is blocked by OPG and anti-inflammatory cytokines such as IL-4 and IL-10, which inhibit inflammation (29). Thus, it is speculated that the increases in both OPG and OPG:RANKL ratio found in the present study could be an anti-inflammatory response to acute phase inflammation that takes place following damage. Similar findings have been reported following myocardial infarction in human subjects (30).

Our results showing increases in IL-6 and OPG and their similar kinetics, combined with the simultaneous decrease in serum RANKL concentrations suggest that these could reflect an anti-inflammatory systemic reaction to muscle damage. Interestingly, previous findings have shown that both IL-6 and OPG inhibit TNF-a – and IL-1 – mediated inflammation (24, 29). Furthermore, our findings appear to be in agreement with suggestions that IL-6 is a key cytokine in the acute phase response, having an inflammation-controlling role and regulating homeostasis following an inflammatory reaction (18, 24, 31, 32). The correlation between increasing concentrations of IL-6 and OPG, as well as the OPG:RANKL ratio post-exercise, could indicate a common modulating role of IL-6 and the OPG/RANKL system during skeletal muscle regeneration following damage. To our knowledge, this is the first report regarding changes in the OPG/RANKL system following exercise-induced muscle damage in humans. Further studies are required to elucidate the potential systemic modulating function of this system following muscle damage. However, it cannot be excluded that the systemic changes in the OPG/RANKL system observed in the present study are not closely associated with the changes occurring within the muscle after damage. Thus, their potential significance regarding muscle inflammation and/or repair process remains to be determined.

In conclusion, we provide the first evidence of the OPG/RANKL system response along with that of IL-6 and other biochemical markers following exercise-induced muscle damage in humans. Clearly, more studies are needed in order to elucidate any systemic and/or local role of the OPG/RANKL system in skeletal muscle inflammation and repair.

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